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Short communication

Soybean *DapA* mutations encoding lysine-insensitive dihydrodipicolinate synthase

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Abstract

In plants, the rate-limiting step in the pathway for lysine synthesis is catalyzed by the enzyme dihydrodipicolinate synthase (DS), which is encoded by the *DapA* gene. We previously cloned the soybean (*Glycine max* cv. Century) *DapA* gene in *Escherichia coli* to express functional soybean DS protein. Like the wild-type soybean DS enzyme, the DS activity encoded by the cloned gene was extremely sensitive to feedback inhibition by micromolar concentrations of lysine. Three mutants of the soybean *DapA* gene were constructed using PCR: one with a single amino acid substitution at codon 104, another with a single amino acid substitution at codon 112, and a mutant containing both modifications. When expressed in *E. coli*, the mutant DS activities were insensitive to lysine at concentrations up to 1 mM.

In plants, the rate-limiting step in the pathway to lysine synthesis is catalyzed by the enzyme dihydrodipicolinate synthase (DS; EC 4.2.1.52). Plant DS is feedback inhibited by lysine and inhibition approaches 100% at lysine levels of less than 100 μM [3, 7, 8, 12, 13]. Many crop plants contain low levels of several essential amino acids. Cereals in particular have limiting amounts of lysine, which must be added to supplement the diets of nonruminant animals. Because of this, the lysine content of plants is an agronomically important trait, and the genetic engineering of DS activity in crop plants has been used to increase their lysine content [4].

In plants, the expression of lysine-insensitive DS activities causes the accumulation of high levels of free lysine [5]. High-lysine tobacco plants with a mutation of the tobacco *DapA* gene were first generated by ethylmethane sulfonate (EMS) mutagenesis of cell cultures and selection using *S*-(2-aminoethyl)L-cysteine (AEC), a toxic lysine analogue [5, 13]. The maize *DapA* gene was later cloned by complementation of an *E. coli* auxotroph and lysine insensitive DS mutants were selected in *E. coli* using AEC [6]. The tobacco [9] and maize [14] lysine-insensitive DS mutants were the result of single-amino-acid substitutions in the DS protein.

Plants can also be genetically engineered to express lysine-insensitive bacterial DS activity. The *E. coli dapA* gene, which encodes DS activity that is relatively insensitive to feedback inhibition, has been expressed in plants [11]. *Corynebacterium glutamicum* DS, which is even less sensitive to lysine, has been expressed in high-lysine crop plants that have been cotransformed to express high-lysine storage proteins [4].

Recently, we cloned the *DapA* gene encoding the soybean (*G. max* cv. Century) DS protein into *E. coli*. The clone expressed high levels of DS activity that was very sensitive to feedback inhibition by lysine, a characteristic of wild-type soybean activity [15]. In this paper, we report the construction of soybean *DapA* clones containing aa substitutions at positions 104

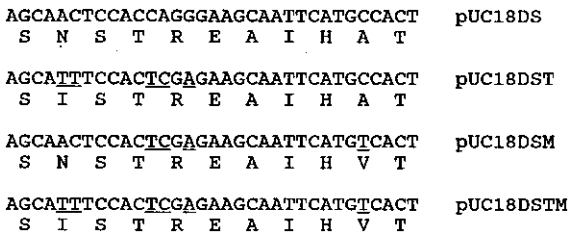

Figure 1. Sequence of *DapA* expression clones corresponding to amino acids 103 to 113 of the mature DS protein expressed *in planta*. Mutations of the nucleotide sequence are underlined. The silent mutation introduces a *Xho*I site. pUC18DS represents wild-type soybean DS, pUC18DST represents soybean DS containing the tobacco mutation Asp→Ile at aa 104, pUC18DSM represents soybean DS containing the maize mutation Ala→Val at amino acid 112, and pUC18DSTM represents soybean DS containing both aa substitutions.

and 112 corresponding to lysine-insensitive mutants of tobacco [9] and maize [14]. When expressed in *E. coli*, the mutant soybean DS activities were insensitive to lysine at concentrations up to 1 mM. A *DapA* mutant with both mutations expressed DS activity with a similar degree of lysine insensitivity. All three mutants expressed high levels of DS activity.

The clone which expresses soybean DS in *E. coli*, pUC18DS, encodes the 326 codons corresponding to the mature DS protein expressed *in planta* [15]. PCR [1] was used to introduce mutations causing the substitution of amino acids at the locations corresponding to amino acid 104 (Asp→Ile) and 112 (Ala→Val) of the mature DS protein (Fig. 1).

Clone pUC18DST expressed soybean DS which Ile is substituted for Asp at codon 104. An Asp→Ile substitution at the corresponding location in tobacco DS made the enzyme lysine-insensitive [9]. To construct pUC18DST, the 5′-proximal end of the *DapA* ORF was amplified from pUC18DS using the vector primer pUC18T7 (AGG AAA CAG CTA TGA CCA TGA TT) and primer 545 (AAT TGC TTC TCG AGT GGA AAT GCT TCC AGT ATT). Primer 545 encodes the amino acid substitution at codon 104 and a silent mutation that adds a *Xho*I site at codon 106. The 3′-proximal end of the *DapA* ORF was amplified using primer 542 (CTG CAG ACT CGA GAA GCA ATT CAT GCC ACT G) and the vector primer 540 (AAG CTT GCA TGC CTG CAG GTC GAC). Primer 542 introduces a silent mutation that adds a *Xho*I site to the DS ORF at codon 106.

Clone pUC18DSM expressed DS in which Val is substituted for Ala at aa 112. An Ala→Val substitution at the corresponding location in maize DS made the enzyme lysine-insensitive [14]. To construct pUC18DSM, the 5′-proximal end of the *DapA* ORF was amplified from pUC18DS using primer pUC18T7 and primer 538 (TGC TTC TCG AGT GGA GTT GCT). Primer 538 introduces a silent mutation that adds a *Xho*I site at codon 106. The 3′-proximal end of the *DapA* ORF was amplified using primer 537 (AAC TCC ACT CGA GAA GCA ATT CAT GTC ACT) and primer 540. Primer 537 encodes the amino acid substitution at codon 112 and a silent mutation that adds a *Xho*I site at codon 106.

Clone pUC18DSTM expressed DS with the amino acid substitutions at both codons 104 and 112 as well as the silent mutation adding the *Xho*I site at codon 106. To construct pUC18DSTM, the 5′-proximal end of the *DapA* ORF was amplified from pUC18DS using primer pUC18T7 and primer 545. The 3′-proximal end of the *DapA* ORF was amplified using primer 537 and primer 540.

PCR cycling conditions were 94 °C for 2 min initial denaturation of genomic DNA, 30 cycles at 94 °C for 2 min, 52 °C for 2 min, and 72 °C for 2 min, followed by a single 7 min final extension at 72 °C.

PCR products encoding the 5′ portion of the *DapA* ORF were cut with *Eco*RI and *Xho*I, while PCR products encoding the 3′ portion of the *DapA* ORF were cut with *Xho*I and *Pst*I. The PCR products were gel isolated and ligated to pUC18 which had been cut with *Eco*RI and *Pst*I [1]. The ligated DNA was used to transform *E. coli* XL1-Blue by electroporation as previously described [15]. *DapA* mutations were sequenced [2], and purified plasmid was used transform the *dapA*$^-$ *E. coli* auxotroph AT997 for the study of DS enzyme activity [15].

Figure 2 shows the effects of lysine on mutant soybean DS activities expressed in *E. coli*. Feedback inhibition of DS was measured in *E. coli* lysates [10]. The *DapA* mutants pUC18DST, pUC18DSM, and pUC18DSTM were assayed, as well as pUC18DS (wild-type soybean DS expressed in *E. coli*). While the wild-type DS activity is very sensitive to feedback inhibition by micromolar concentrations of free lysine, the mutant soybean DS activities are insensitive to inhibition by free lysine at a concentration of 1.0 mM.

Our results demonstrate the functional similarities of the DS enzymes of different plant species. Soybean DS was made lysine-insensitive by single-amino-acid substitutions corresponding to lysine-insensitive DS tobacco and maize mutants. The mutations of codons 104 and 112 of soybean DS produce similar resistance to feedback inhibition. The feedback inhibition of soy-

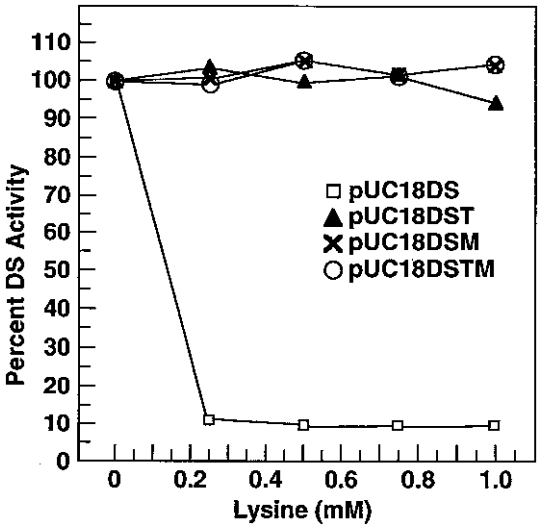

Figure 2. Lysine inhibiton of mutant and wild-type *G. max* DS activity expressed by *E. coli dapA*$^-$ auxotrophic cells. pUC18DS (□) wild-type soybean DS, pUC18DST (▲) Asp→Ile at amino acid 104, pUC18DSM (X) Ala→Val at 112, pUC18DSTM (○) Asp→Ile at 104 and Ala→Val at 112

bean DS containing both mutations is indistinguishable from either of the single mutants (Fig. 2), and the DS activity of all the mutants is expressed at similar levels in *E. coli* (data not shown).

The mutations which were introduced in soybean *DapA* are located within an aa sequence that is conserved in monocot and dicot DS proteins [14] and the corresponding mutations make tobacco and maize DS insensitive to feedback inhibition by lysine. This suggests that the conserved region of DS is essential for feedback inhibition by lysine. The similarity of the lysine insensitive DS activities expressed by pUC18DST, pUC18DSM, pUC18DSTM is consistent with a mechanism for feedback inhibition that is conserved in both monocots and dicots.

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